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ORIGINAL ARTICLE

Properties of putrescine uptake by PotFGHI and PuuP and their physiological significance in *Escherichia coli*

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Abstract Properties of putrescine uptake by PotFGHI and PuuP and their physiological significance were studied using a polyamine biosynthesis and uptake deficient *Escherichia coli* KK3131 transformed with pACYC184 containing *potFGHI* or *puuP*. Putrescine uptake activity of *E. coli* KK3131 transformed with pACYC184-PotFGHI was higher than that of *E. coli* 3131 transformed with pACYC-PuuP when cells were cultured in the absence of putrescine. Putrescine uptake by PotFGHI was both ATP and membrane potential dependent, while that by PuuP was membrane potential dependent. Feedback inhibition by polyamines occurred at the PotFGHI uptake system but not at the PuuP uptake system. Expression of PuuP was reduced in the presence of PuuR, a negative regulator for PuuP, and expression of PuuR was positively regulated by glucose, which reduces the level of cAMP. The complex of cAMP and CRP (cAMP receptor protein) inhibited the expression of PuuR in the absence of glucose. Thus, the growth rate of *E. coli* KK3131 in the presence of both 0.4 % (22.2 mM) glucose and 10 mM putrescine was in the order of cells transformed with pACYC-PotFGHI > pACYC-PuuP > pACYC-PuuP + PuuR, which was parallel with the polyamine content in cells. The results indicate that PotFGHI is necessary for rapid cell growth in the presence of glucose as an energy source. When glucose in medium was depleted, however, PuuP was absolutely necessary for cell growth in the presence of putrescine, because accumulation of putrescine to a high level by PuuP was necessary for utilization of putrescine as an energy source.

Keywords Putrescine · Transport · Feedback inhibition · Energy source · cAMP

Abbreviations

CadB	Cadaverine-lysine antiporter
CCCP	Carbonyl cyanide *m*-chlorophenylhydrazone
CRP	cAMP receptor protein
PotE	Putrescine-ornithine antiporter

Introduction

Polyamines (putrescine, spermidine, and spermine) are essential for normal cell growth (Cohen 1998; Igarashi and Kashiwagi 2010b), and their content in cells is regulated by biosynthesis, degradation, uptake, and excretion (Casero and Pegg 2009; Igarashi and Kashiwagi 2010a, b; Marton and Pegg 1995). With regard to transport, the properties of three polyamine transport systems were characterized in *Escherichia coli* (Higashi et al. 2010; Igarashi and Kashiwagi 2010a; Kashiwagi et al. 1990). They include spermidine-preferential (PotABCD) and putrescine-specific (PotFGHI) uptake systems, which belong to the family of

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ATP binding cassette transporters, and a protein, PotE, involved in the excretion of putrescine at acidic pH by a putrescine-ornithine antiporter activity. Furthermore, it has been reported that cadaverine and aminopropylcadaverine function as compensatory polyamines for cell growth (Igarashi et al. 1986), and CadB, a cadaverine-lysine antiporter, is strongly involved in cell growth at acidic pH, like PotE (Neely and Olson 1996; Soksawatmaekhin et al. 2004; Tomitori et al. 2012; Watson et al. 1992). Analogous to the *speF-potE* operon (Kashiwagi et al. 1991), *cadB* is one component of the *cadBA* operon, in which *cadA* encodes lysine decarboxylase (Meng and Bennett 1992; Neely and Olson 1996) and is induced by acidic pH and lysine (Soksawatmaekhin et al. 2004). The *speF-potE* and *cadBA* operons contribute to an increase in the pH of the extracellular medium through excretion of putrescine and cadaverine, the consumption of protons to create the membrane potential, and a supply of carbon dioxide for nucleotide synthesis during the decarboxylation reaction (Takayama et al. 1994), hence the expression of these two operons is important for cell growth at acidic pH. A spermidine excretion protein complex (MdtJI) whose expression is enhanced by spermidine has also been identified (Higashi et al. 2008). Thus, polyamine content is well regulated by many polyamine transport systems to maintain optimal concentrations necessary for cell growth in the presence of glucose as an energy source.

Recently, it has been reported that PuuP functions as putrescine uptake protein when putrescine is used as an energy source (Kurihara et al. 2009). Thus, putrescine uptake by PotFGHI and PuuP was compared using *E. coli* cells deficient in polyamine biosynthesis and uptake, in the presence of glucose and putrescine as energy sources. It was found that PotFGHI functions more effectively than PuuP for the stimulation of cell growth by polyamines in the presence of glucose, and PuuP is necessary for cell growth in the presence of putrescine and absence of glucose.

Materials and methods

Bacterial strains and culture conditions

Escherichia coli KK3131 [old name KK313 *potF::Km*], which is deficient in both putrescine biosynthesis and putrescine and spermidine uptake, was prepared from putrescine biosynthesis deficient *E. coli* MA261 (*speB speC serA thr leu thi*) (Cunningham-Rundles and Maas 1975) as described previously (Kashiwagi et al. 2002; Pistocchi et al. 1993). *E. coli* KK3131 was deficient in PotABCD [Val-135 is mutated to Met in PotA ATPase (Kashiwagi et al. 2002)], PotFGHI (*potF::Km*) and PuuP [Tyr-110 converted to Cys (Kurihara et al. 2009)] activities. Putrescine can be converted to spermidine in *E. coli* KK3131. *E. coli* MA261 *Δcya, crp* [old name MA261 *cya::Km crp::Tet*] was prepared as described previously (Yoshida et al. 2001). The cells were cultured overnight at 37 °C in Luria–Bertani (LB) medium (10 g of tryptone, 5 % of yeast extract and 10 g of NaCl per liter) containing kanamycin (0.05 mg/ml), chloramphenicol (0.03 mg/ml) and/or tetracycline (0.015 mg/ml), transferred to medium A (40.2 mM K_2HPO_4, 22.1 mM KH_2PO_4, 1.7 mM sodium citrate, 7.6 mM $(NH_4)_2SO_4$, 0.41 mM $MgSO_4$, 6 μM thiamine, 40 μM biotin, 0.8 mM threonine, 0.7 mM methionine, 1 mM serine, 1 mM glycine, 0.6 mM ornithine, pH 6.8) with 0.4 % (22.2 mM) glucose, and grown till A_{540} reached 1.0. Then, *E. coli* cells were cultured at an A_{540} of 0.1 and growth was monitored at 37 °C by measuring A_{540} in medium A containing 0 or 10 mM putrescine with either 0.4 % (22.2 mM) or 0.04 % (2.22 mM) glucose.

Plasmids

A medium-copy number vector pACYC184 was used for construction of pACYC-PuuP and pACYC-PuuP + PuuR. PCRs were performed using total chromosomal DNA from *E. coli* W3110 as a template, and P1 (5′-GGAGCCAG GATCCACATTTGCTGGTAATCC-3′) and P2 (5′-TTA TTTGCGGATCCGGTTAATGCGCTAAGC-3′) as primers for amplification of *puuP* and *puuA* genes, P3 (5′-GCATAAGGGCGGATCCTAGCACTCGTGATC-3′) and P4 (5′-TAAGCTCATCGGGATCCGCTCACTTGCCG-3′) as primers for amplification of *puuP* + *puuR* genes (*puuP puuA puuD puuR*) (Kurihara et al. 2008). The amplified genes were digested with BamHI and inserted into the restriction site of pACYC184. Plasmid pACYC-PotFGHI (old name, pPT79) was prepared as described previously (Kashiwagi et al. 1990). A high-copy number vector pET-52b(+) (Novagen) was used for construction of pET-PuuP(His) and pET-PotFGHI(His). PCRs were performed using total chromosomal DNA from *E. coli* W3110 as a template, and P5 (5′-GGAGCCAGGATCCACATTTGC TGGTAATCC-3′) and P6 (5′-ATTGCGGCCGCCGTTT CACTCACCGGCGTT-3′) as primers for amplification of *puuP* gene, P7 (5′-AAGTGCAGGATCCGAAAATCGA CCGTGTGC-3′) and P8 (5′-TATGCGGCCGCGCCACG TCTTGCACGCTGG-3′) as primers for amplification of *potFGHI* genes. The amplified genes were digested with BamHI and NotI, and inserted into the restriction site of pET-52b(+). In case of pACYC-PuuP(His), a PCR was performed using pET-PuuP as template and P5 and P9 (5′-GGCAGGATCCTAGGTTAATTAGTGGTGGTG-3′) as primers. The BamHI fragment containing *puuP* gene with a His_6 tag was inserted into the same restriction site of pACYC184. Plasmid pACYC-PuuP(His) + PuuR was

constructed by exchanging the SalI fragment of pACYC-PuuP + PuuR with the SalI fragment of *puuP(His)* gene of pET-PuuP(His). In case of pACYC-PotFGHI(His) containing *potI* gene with a His_6 tag at COOH terminus, a PCR was performed using pET-PotFGHI(His) as template and P10 (5′-TGCGGTACCTGCAAGCTTCAAAATTATCTG-3′) and P11 (5′-GGAAGCTTCCTAGGTTAATTAGTGG TGGTG-3′) as primers. The HindIII fragment containing *potFGHI(His)* gene was inserted into the same restriction site of pACYC184. The nucleotide sequence of the plasmids was confirmed with a CEQ8000 DNA genetic analysis system (Beckman Coulter). Transformation of *E. coli* KK3131 with these plasmids was carried out by electroporation (Sambrook and Russell 2001). Colonies of transformants with pACYC184 were grown on LB agar plate containing kanamycin (0.05 mg/ml), chloramphenicol (0.03 mg/ml) and/or tetracyclin (0.015 mg/ml).

Putrescine uptake by intact cells

Escherichia coli KK3131 cells carrying pACYC-PotFGHI or pACYC-PuuP were cultured in 100 ml medium A containing 0.4 % glucose in the absence of putrescine until $A_{540} = 0.5$, and were suspended in medium A containing 0.4 % glucose to yield a protein concentration of 0.03–0.1 mg/ml. The cell suspension (0.48 ml) was pre-incubated at 30 °C for 5 min, and the reaction was started by the addition of 0.02 ml of 0.5 mM [^{14}C]putrescine (370 MBq/mmol, Sigma). Thus, the concentration of [^{14}C]putrescine in the reaction mixture was 20 μM. After incubation at 30 °C for 5 min, the cells were collected on cellulose acetate filters (0.45 μM; Advantec Toyo Co.), and the radioactivity on the filters was measured with a liquid scintillation spectrometer. Protein content was determined by the method of Bradford (Bradford 1976) after trichloroacetic acid precipitation of cells.

Dot blot and Western blot analysis

Escherichia coli KK3131 cells carrying pACYC-PotFGHI(His), pACYC-PuuP(His) or pACYC-PuuP(His) + PuuR were cultured in 100 ml medium A containing 0.4 or 0.04 % glucose with 10 mM putrescine until $A_{540} = 0.5$. Total RNA was prepared from these cells by the method of Emory and Belasco (Emory and Belasco 1990). Dot blot analysis was performed using ECL direct nucleic acid labeling and detection system (GE Healthcare Bio-Sciences) according to the accompanying manual. Probes were made by PCR, and the sizes of the probes used for *puuP*, *puuR* and *potFGHI* were 756, 432, and 1,263 nucleotides, respectively. Chemical luminescence was detected by a LAS-1000 plus luminescent image analyzer (Fuji Film).

Western blot analysis was performed using ECL Western blotting reagents (GE Healthcare Bio-Sciences). Membrane protein fractions were prepared as described previously (Oliver and Beckwith 1982). Approximately 2–3 mg inner membrane protein fraction was obtained when cells were cultured in 100 ml medium A containing 0.4 % glucose. The amount of proteins used for Western blotting was described in the legends of figures. Anti-His(C-term) antibody was obtained from Invitrogen, and antibody against chloramphenicol acetyltransferase was from Abcam. Antibodies against CRP and RNA polymerase σ^{70} subunit were kindly provided by Dr. A. Ishihama at Hosei University. The level of protein on the blot was quantified with a LAS-3000 luminescent image analyzer (Fuji Film).

Measurement of polyamines, ATP and cAMP in cells

Polyamines and ATP were extracted by treatment of the cells with 10 % trichloroacetic acid with occasional shaking. Polyamine content was determined by high-pressure liquid chromatography as described previously (Igarashi et al. 1986). ATP content was determined by ENLITEN® ATP Assay System Bioluminescence Detection Kit for ATP measurement (Promega) according to the accompanying manual. The cAMP content in cells was measured using a cAMP enzyme immunoassay system (GE Healthcare Bio-Sciences) as described previously (Terui et al. 2007).

Results

Putrescine uptake by PotFGHI and PuuP

The genes encoding proteins for putrescine uptake—PotFGHI and PuuP—and for PuuR, a negative regulator of *puuP* gene expression (Kurihara et al. 2008; Nemoto et al. 2012; Pistocchi et al. 1993) are illustrated in Fig. 1a. Putrescine uptake in polyamine-deficient KK3131 cells transformed with pACYC-PotFGHI or with pACYC-PuuP was compared under the same experimental conditions. As shown in Fig. 1b, the uptake of putrescine by PotFGHI was greater than that by PuuP. Furthermore, the uptake of putrescine by PotFGHI was decreased in the absence of glucose and by the addition of 40 μM CCCP (carbonyl cyanide *m*-chlorophenylhydrazone), an inhibitor of proton circulation, indicating that putrescine uptake by PotFGHI is both ATP and membrane potential dependent, similar to spermidine uptake by PotABCD (Kashiwagi et al. 2002). In contrast, the uptake of putrescine by PuuP was dependent on the membrane potential, because it was inhibited by 40 μM CCCP, but the decrease in ATP did not inhibit

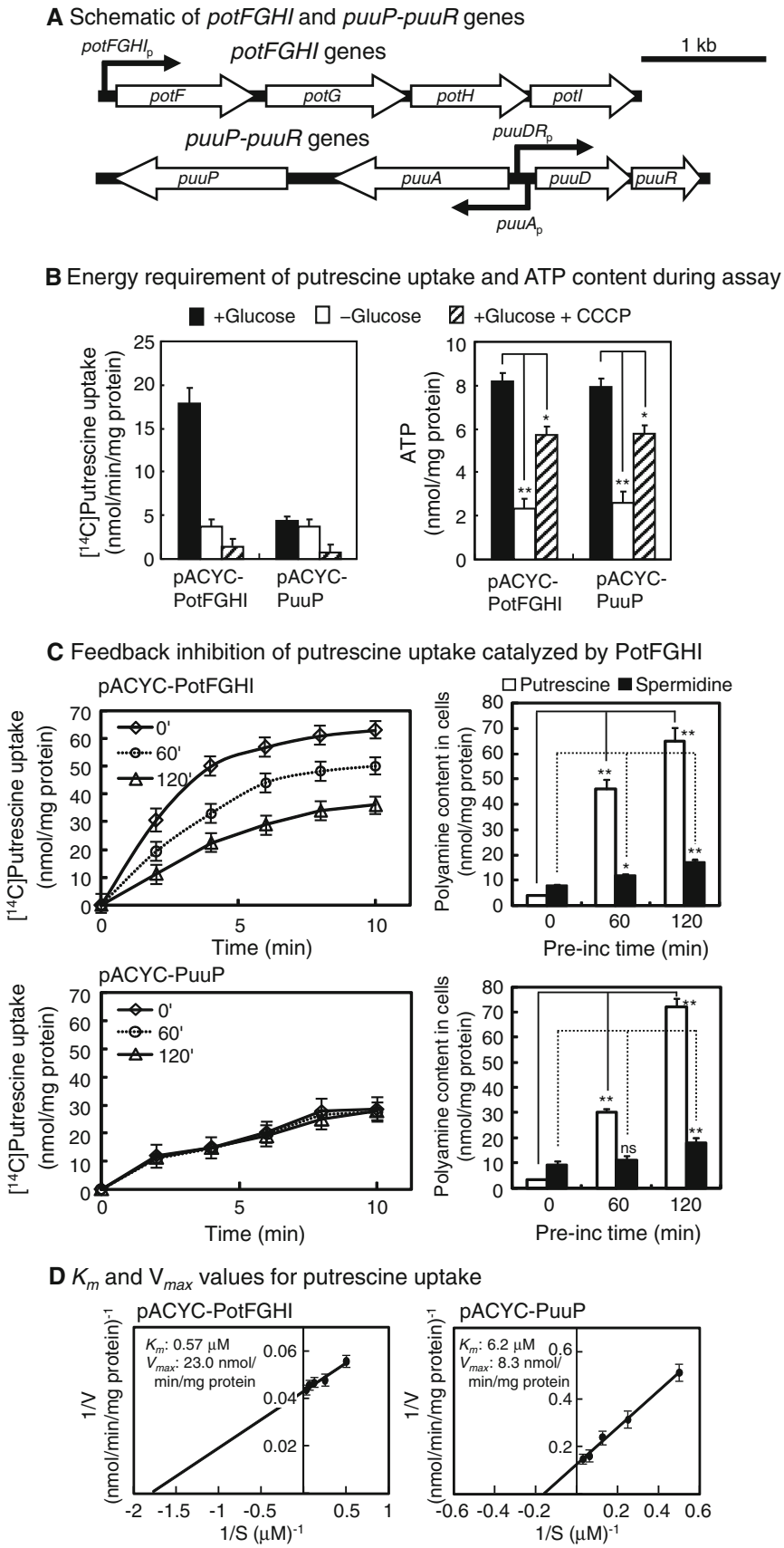

Fig. 1 Putrescine uptake activity by PotFGHI and PuuP. **a** Schematic of *potFGHI* and *puuP-puuR* genes is shown (Kurihara et al. 2008; Nemoto et al. 2012; Pistocchi et al. 1993). The *puuA* and *puuD* genes encode γ-glutamylputrescine synthetase and γ-glutamyl-γ-aminobutyrate hydrolase, respectively. Initiation points of transcription are shown by *arrows*. **b** Putrescine uptake was measured in medium A with or without 0.4 % glucose and 40 μM CCCP during the incubation time. Putrescine uptake activity was shown by subtracting putrescine uptake activity of cells transformed with pACYC184 (0.21 nmol/min/mg protein) as a background. ATP content in cells was measured as described in "Materials and methods" after 5 min incubation in the putrescine uptake assay mixture containing 20 μM nonlabeled putrescine with or without 0.4 % glucose and 40 μM CCCP. **c** *Escherichia coli* cells were cultured until $A_{540} = 0.4$, and putrescine was added to the medium to make the concentration at 10 mM. At 0, 60 and 120 min incubation, cells were harvested, washed with putrescine-free medium three times, and putrescine uptake activity (*left figures*) and polyamine content in cells (*right figures*) were measured as described in "Materials and methods". **d** The K_m and V_{max} values were calculated from the Lineweaver-Burk plot. Values of putrescine uptake activity, ATP and polyamine content are means ± standard errors of triplicate determinations. *ns*, $p \geq 0.05$, $^*p < 0.05$, $^{**}p < 0.01$

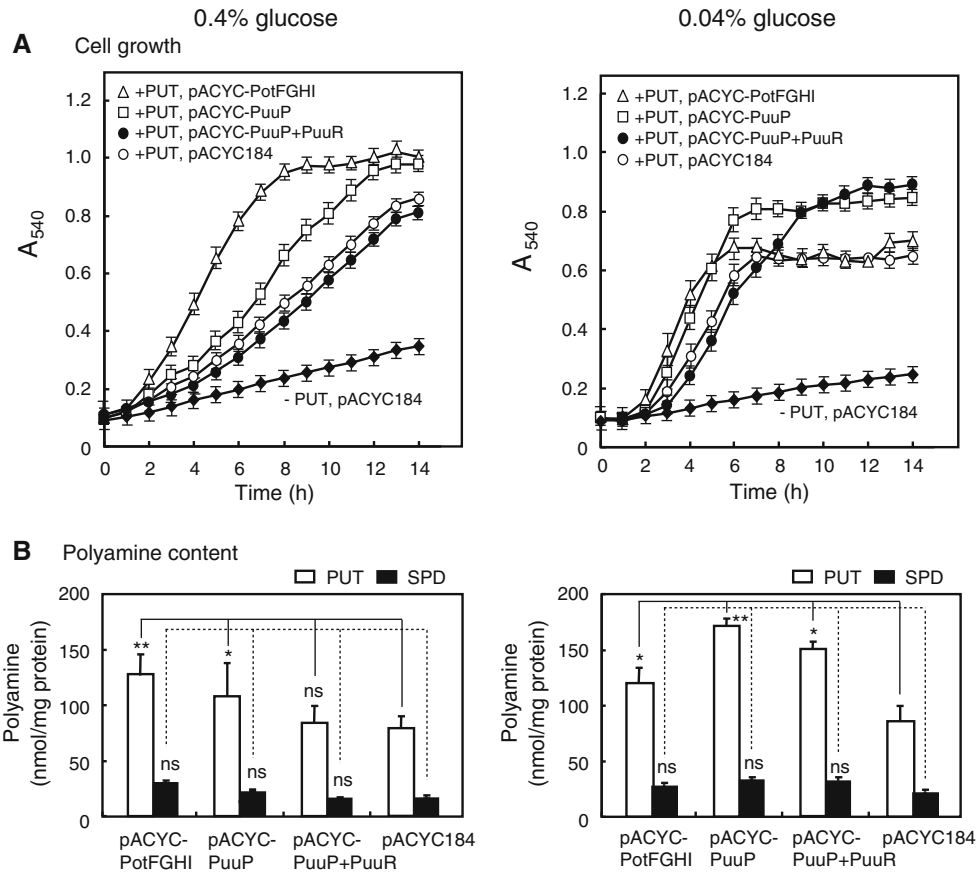

Fig. 2 Effect of 0.4 % (22.2 mM) or 0.04 % (2.22 mM) glucose and 10 mM putrescine on growth of polyamine-deficient cells transformed with pACYC184 encoding various putrescine transporters. **a** Cell growth was followed by measuring A_{540}. *Open triangle E. coli* KK3131/pACYC-PotFGHI, *open box E. coli* KK3131/pACYC-PuuP, *filled circle E. coli* KK3131/pACYC-PuuP + PuuR, *open circle* and *filled diamond E. coli* KK3131/pACYC184 vector. Cells were cultured in the presence of 0.4 or 0.04 % glucose with 10 mM putrescine except *E. coli* KK3131/pACYC184 (*filled diamond*) as shown in the figure. *Values* are means ± standard errors of triplicate determinations. *PUT* putrescine. **b** Polyamine content in cells harvested at $A_{540} = 0.5$ were measured as described in "Materials and methods". *Values* are means ± standard errors of triplicate determinations. *ns*, $p \geq 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$

putrescine uptake by PuuP (Fig. 1b). Uptake of putrescine by PotFGHI and PuuP was not inhibited by diaminopropane, cadaverine, spermidine, and γ-aminobutyric acid, an intermediate of putrescine utilization pathway (Kurihara et al. 2008), indicating that both systems are putrescine-specific (data not shown).

We have reported that spermidine uptake by PotABCD was inhibited by spermidine through its interaction with the COOH-terminal domain of PotA ATPase (Kashiwagi et al. 2002). Thus, it was determined whether feedback inhibition occurs for putrescine uptake by PotFGHI and PuuP. As shown in Fig. 1c, putrescine uptake by PotFGHI was gradually inhibited by increasing levels of intracellular polyamines (putrescine and spermidine), whereas the activity of PuuP was not affected by intracellular polyamine levels. These results suggest that putrescine uptake by PotFGHI can contribute to the maintenance of optimal polyamine levels in cells through feedback inhibition by accumulated polyamines, analogous to feedback inhibition of spermidine uptake by PotABCD. Then, the K_m values for putrescine and the V_{max} values for putrescine uptake of PotFGHI and PuuP were compared under the same experimental conditions. The K_m values for putrescine uptake by PotFGHI and PuuP were 0.57 and 6.2 μM, respectively, and the V_{max} values were 23.0 and 8.3 nmol/min/mg protein, respectively (Fig. 1d). The results were similar to the previous reports (Kashiwagi et al. 1990; Kurihara et al. 2009) except the V_{max} value for PuuP. This suggests that the level of PuuP synthesis may change according to the nutritional conditions of cell culture.

Participation of PotFGHI and PuuP in cell growth of putrescine biosynthesis- and uptake-deficient *E. coli* KK3131 cells

The effects of PotFGHI and PuuP on cell growth were determined in the presence of 0.4 % (22.2 mM) glucose and 10 mM putrescine using cells transformed with pACYC184-PotFGHI, pACYC-PuuP and pACYC-PuuP + PuuR. As shown in Fig. 2a, cell growth was in the order pACYC-PotFGHI > pACYC-PuuP > pACYC-PuuP + PuuR ≈ pACYC184 vector. The cell growth of *E. coli* KK3131/pACYC-PuuP + PuuR was nearly equal to that of *E. coli* KK3131/pACYC184 vector, indicating that putrescine uptake system catalyzed by PuuP does not function in the presence of PuuR. The generation time of *E. coli* KK3131 cells transformed with pACYC-PotFGHI, pACYC-PuuP and pACYC-PuuP + PuuR was 90 min, 180 and 240 min, respectively. The polyamine content in cells was parallel with the rate of cell growth: i.e. levels of

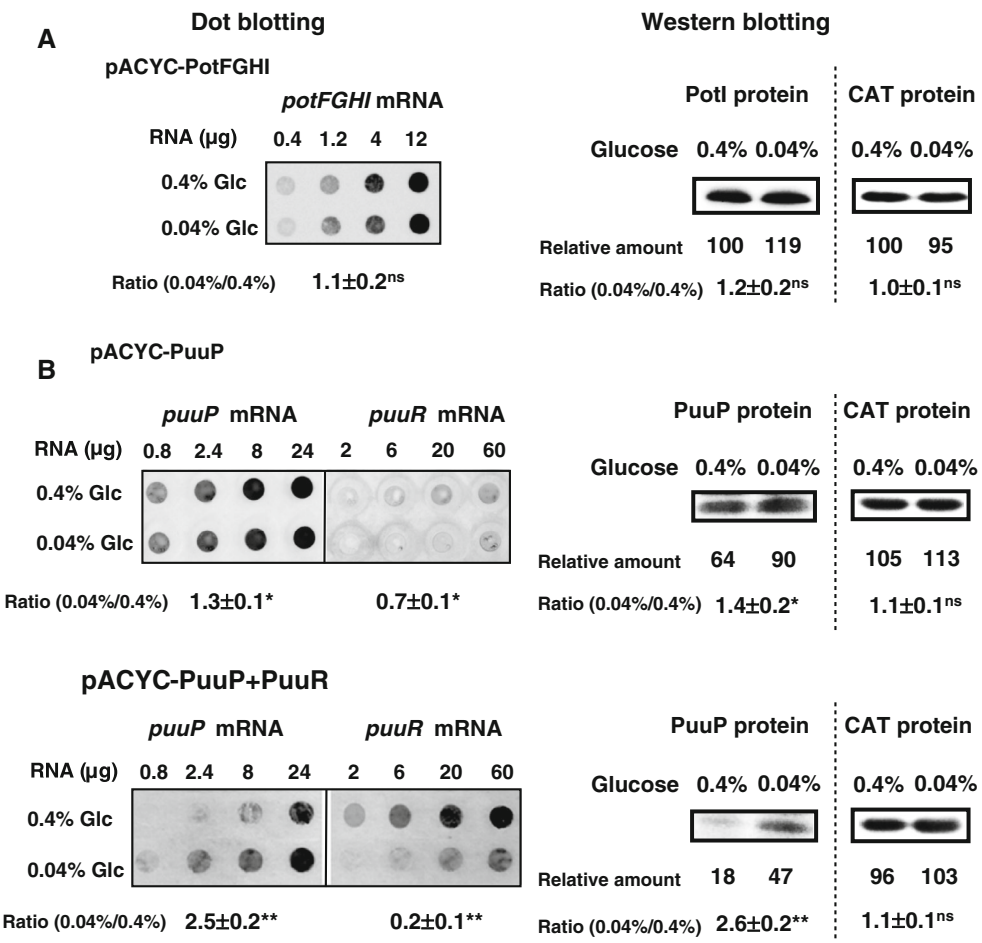

Fig. 3 Measurement of PotI and PuuP protein level and *potFGHI*, *puuP* and *puuR* mRNA level under various conditions. *E. coli* KK3131 cells transformed with pACYC-PotFGHI(His), pACYC-PuuP(His) and pACYC-PuuP(His) + PuuR were cultured in the presence of 0.4 % (22.2 mM) or 0.04 % (2.22 mM) glucose with 10 mM putrescine until $A_{540} = 0.5$. Western blotting of PotI-His and PuuP-His was performed using 30 μg proteins and anti-His(C-term) antibody. The level of chloramphenicol acetyltransferase (CAT) was measured as a control using 5 μg protein. Dot blotting for *potFGHI* mRNA, *puuP* mRNA and *puuR* mRNA was performed as described in "Materials and methods". *Values* are means ± standard errors of triplicate determinations. *ns*, $p \geq 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$

putrescine and spermidine in cells transformed with pACYC-PotFGHI, pACYC-PuuP, pACYC-PuuP + PuuR and pACYC184 vector were 128 and 30, 108 and 22, 78 and 16, and 76 and 15 nmol/mg protein, respectively, when cells were harvested at $A_{540} = 0.5$ (Fig. 2b). The results indicate that the putrescine uptake by PotFGHI is more effective than that by PuuP in the presence of glucose, and confirmed that PuuR is an effective negative regulator for PuuP expression.

Then, the effects of PotFGHI and PuuP on cell growth were determined in the presence of 0.04 % (2.22 mM) glucose and 10 mM putrescine. As shown in Fig. 2a, cell growth was in the order pACYC-PotFGHI ≈ pACYC-PuuP > pACYC-PuuP + PuuR ≈ pACYC184 vector. When glucose became deficient in the medium, growth of cells transformed with pACYC-PotFGHI and pACYC184 vector stopped, but that of cells transformed with pACYC-PuuP and pACYC-PuuP + PuuR continued until putrescine became depleted (Fig. 2a). The results indicate that putrescine is used as an energy source only when PuuP functions as a putrescine importer. Polyamine content in cells harvested at $A_{540} = 0.5$ was nearly parallel with the degree of maximal cell growth. Putrescine and spermidine content in cells transformed with pACYC-PuuP was 174 and 32 nmol/mg protein, respectively, which are estimated to be 60 and 11 mM, respectively, if *E. coli* cell volume is estimated to be 2.9 μl/mg protein (Bakker and Mangerich 1981), while the putrescine and spermidine content in cells transformed with pACYC-PotFGHI was 115 and 22 nmol/mg protein, respectively, which are estimated to be 40 and 7.6 mM, respectively (Fig. 2b). Polyamine content in *E. coli* KK3131/pACYC-PuuP + PuuR was higher than that in *E. coli* KK3131/pACYC-PotFGHI, because PuuP functioned in the absence of glucose. These results support the idea that accumulation of a high concentration of putrescine is necessary for putrescine utilization as an energy source.

Comparison of expression level of PotFGHI and PuuP

The levels of PotFGHI and PuuP proteins and those of *potFGHI* and *puuP* mRNAs were analyzed by Western blotting and dot blot analysis, respectively. As shown in Fig. 3, both *potFGHI* mRNA and protein levels were approximately 1.6-fold higher than *puuP* mRNA and protein levels. The results indicate that rapid putrescine uptake

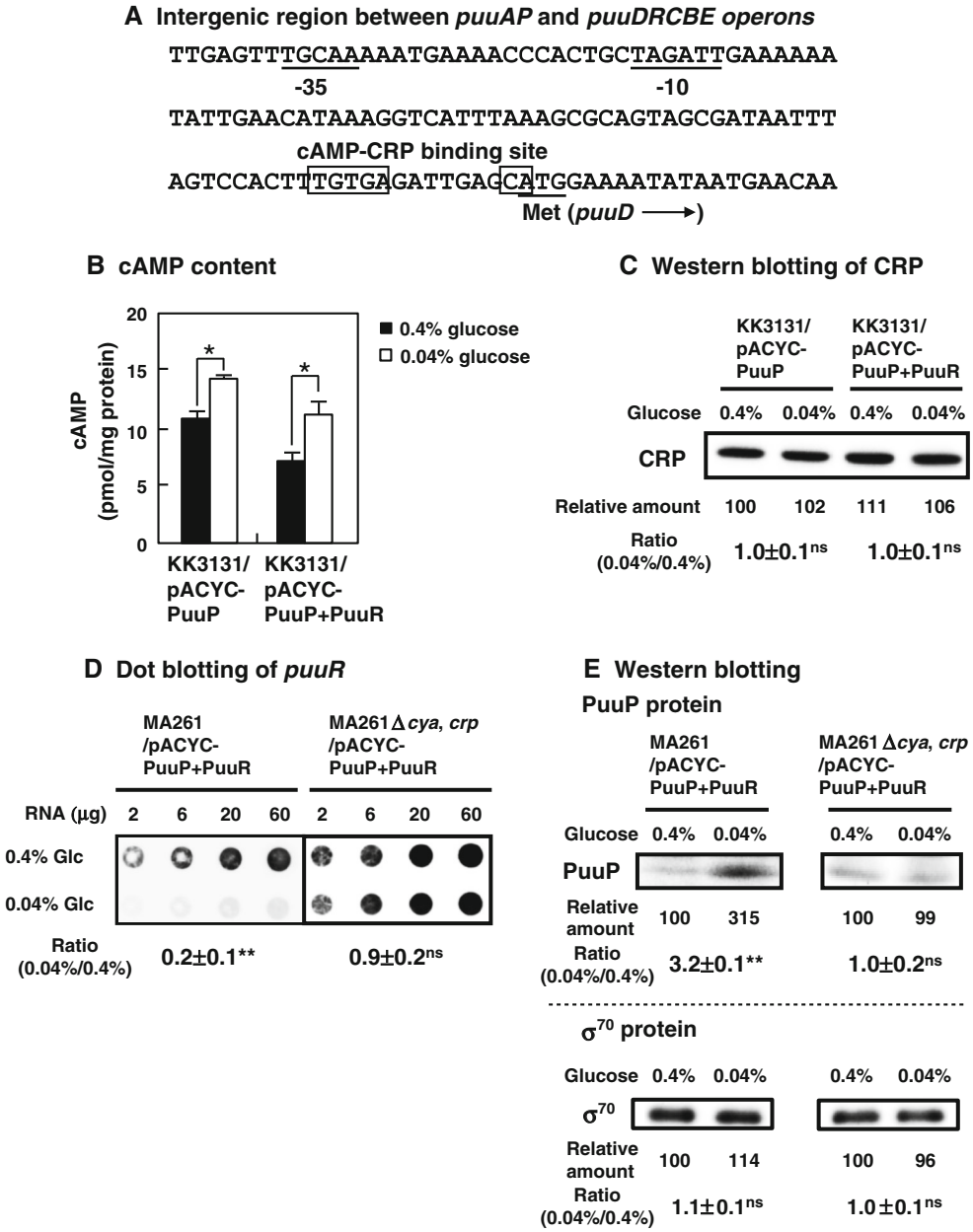

Fig. 4 Regulation of PuuP synthesis by PuuR repressor through the cAMP-CRP protein complex. **a** Nucleotide sequence of intergenic region between *puuAP* operon and *puuDRCBE* operon (Nemoto et al. 2012) was shown. **b** and **c** The levels of cAMP and CRP in *E. coli* KK3131/pACYC-PuuP and *E. coli* KK3131/pACYC-PuuP + PuuR were measured as described in "Materials and methods". The level of cAMP in *E. coli* KK3131/pACYC-PuuP was significantly higher than that in *E. coli* KK3131/pACYC-PuuP + PuuR. **d** and **e** Dot blotting of *puuR* mRNA and Western blotting of PuuP protein in *E. coli* MA261 *Δcya, crp* and its parent strain *E. coli* MA261 were performed as described in "Materials and methods". Western blotting of CRP and PuuP was performed using 30 μg protein, and that of RNA polymerase σ^{70} subunit was performed using 2 μg protein. *Values* are means ± standard errors of triplicate experiments. *ns*, $p \geq 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$

by PotFGHI was mainly due to the high level expression of PotFGHI, rather than PuuP. It was also shown that the level of PuuP protein was higher when glucose concentration was low, which roughly reflects the level of *puuP* mRNA. When *puuP* was expressed together with *puuR* in the presence of 0.4 % glucose, the level of PuuP protein was repressed by PuuR, which was parallel with the level of *puuP* mRNA. Under these conditions the level of *puuR* mRNA was high. The results suggest that expression of *puuR* mRNA is positively regulated by glucose, so that expression of *puuP* mRNA is negatively regulated by glucose.

The inhibitory mechanism of expression of *puuR* mRNA at low concentration (0.04 %) of glucose was then studied.

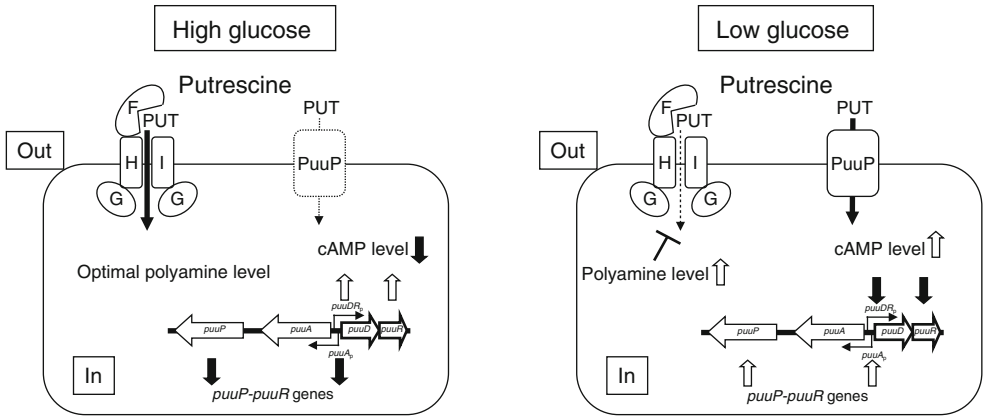

Fig. 5 Physiological functions of PotFGHI and PuuP in the presence of 0.4 % (22.2 mM) or 0.04 % (2.22 mM) glucose with 10 mM putrescine. In the presence of 0.04 % glucose, expression of PuuP was enhanced through repression of PuuR expression, and PotFGHI activity was inhibited by accumulated polyamines

There is a cAMP-CRP recognition site (Shimada et al. 2011) between the initiation site of transcription and the initiation codon ATG in *puuD* gene in the *puuDRCBE* operon (Figs. 1a, 4a). Thus, if the level of cAMP is high in cells cultured in the presence of 0.04 % glucose, negative regulation by the cAMP-CRP complex probably occurs in the *puuR* gene expression (Shimada et al. 2011). As shown in Fig. 4b and c, the level of cAMP was higher when cells were cultured in the presence of 0.04 % glucose compared to 0.4 % glucose, while the level of CRP was nearly equal in cells cultured in the presence of either 0.4 or 0.04 % glucose. Stimulation of PuuP synthesis by cAMP through inhibition of *puuR* expression was confirmed using *E. coli* MA261 *Δcya, crp* and its parent strain. As shown in Fig. 4d and e, expression of *puuR* gene was greatly enhanced in *E. coli* MA261 *Δcya, crp* compared to its parent strain, and accordingly, synthesis of PuuP protein was strongly inhibited even at low concentration (0.04 %) of glucose. These results supports the idea that synthesis of PuuP at low concentration of glucose was enhanced through inhibition of *puuR* expression, and that the level of cAMP is important for negative regulation of *puuR* expression in the presence of 0.04 % glucose.

Discussion

In *E. coli*, two major putrescine-specific uptake systems, i.e. PotFGHI and PuuP, have been reported (Kurihara et al. 2008; Pistocchi et al. 1993). Properties and physiological functions of both uptake systems were studied using a polyamine biosynthesis and uptake deficient *E. coli* KK3131 transformed with pACYC-PotFGHI or pACYC-PuuP. Results obtained in this study indicate that both PotFGHI and PuuP contribute to the growth of polyamine-deficient cells cultured in the presence of 0.4 % (22.2 mM) glucose and 10 mM putrescine. Under these conditions, PotFGHI enhanced cell growth more effectively than PuuP, because the expression level of PotFGHI was higher than that of PuuP and the K_m value for putrescine was lower in PotFGHI than in PuuP. In culture conditions where putrescine became a major energy source, PuuP was required as the putrescine transporter. Putrescine transport by PuuP was not inhibited by the accumulated polyamines, so that high concentrations of putrescine could be accumulated to synthesize γ-glutamylputrescine, the first intermediate for putrescine utilization as an energy source. The K_m value of putrescine for γ-glutamylputrescine synthetase was 44.6 mM (Kurihara et al. 2008). Accumulation of a high concentration of putrescine did not occur with PotFGHI, because of feedback inhibition of putrescine uptake by polyamines. Thus, PuuP is essential for putrescine utilization as an energy source.

When PuuP was expressed in the presence of PuuR, cell growth was delayed, because PuuR is a negative regulator of PuuP expression (Nemoto et al. 2012). It also became clear that PuuR expression is positively regulated by glucose, which decreases the level of cAMP. The transcription of *puuR* mRNA is most likely inhibited through the binding of cAMP-CRP complex to transcription initiation region of the *puuDRCBE* operon in the presence of 0.04 % (2.22 mM) glucose (see Fig. 4d). Similar negative regulation of the level of *ompA* mRNA by cAMP-CRP complex has been recently reported (Lin et al. 2011). It has been reported that putrescine uptake by PuuP was higher than that by PotFGHI using the polyamine-containing cells (Kurihara et al. 2009). This may be explained by the finding that putrescine uptake by PotFGHI, but not by PuuP, was inhibited by accumulated polyamines (see Fig. 1c), and that the level of PuuP expression was increased in the presence of low concentration of glucose (see Fig. 3b). If putrescine content in cells increased

further (see Fig. 2b), it is expected that putrescine uptake by PotFGHI decreased further.

Judging from the results shown in Fig. 2a, there is another putrescine uptake system in addition to PotFGHI, PuuP and PotABCD. Experiments are now in progress to find another gene encoding a new putrescine uptake protein. When *E. coli* KK3131 was transformed with low copy number plasmid pMW-PotFGHI, pMW-PuuP and pMW-PuuP + PuuR, a similar dependency of cell growth was also observed in the presence of 0.4 % (22.2 mM) glucose or 0.04 % (2.22 mM) glucose with 10 mM putrescine (data not shown).

The results obtained in this study clearly indicate the physiological function of two putrescine uptake systems: PotFGHI imports putrescine for maintenance of the optimal concentration of polyamines necessary for cell growth in the presence of glucose, whereas PuuP imports putrescine for its utilization as an energy source in the absence of glucose. The physiological functions of PotFGHI and PuuP were summarized in Fig. 5.

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Conflict of interest The authors declare that they have no conflict of interest.

References

Bakker EP, Mangerich WE (1981) Interconversion of components of the bacterial proton motive force by electrogenic potassium transport. J Bacteriol 147:820–826

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Casero RA, Pegg AE (2009) Polyamine catabolism and disease. Biochem J 421:323–338

Cohen SS (1998) A guide to polyamines. Oxford University Press, New York

Cunningham-Rundles S, Maas WK (1975) Isolation, characterization, and mapping of *Escherichia coli* mutants blocked in the synthesis of ornithine decarboxylase. J Bacteriol 124:791–799

Emory SA, Belasco JG (1990) The *ompA* 5′ untranslated RNA segment functions in *Escherichia coli* as a growth-rate-regulated mRNA stabilizer whose activity is unrelated to translational efficiency. J Bacteriol 172:4472–4481

Higashi K, Ishigure H, Demizu R, Uemura T, Nishino K, Yamaguchi A, Kashiwagi K, Igarashi K (2008) Identification of a spermidine excretion protein complex (MdtJI) in *Escherichia coli*. J Bacteriol 190:872–878

Higashi K, Sakamaki Y, Herai E, Demizu R, Uemura T, Saroj SD, Zenda R, Terui Y, Nishimura K, Toida T, Kashiwagi K, Igarashi K (2010) Identification and functions of amino acid residues in PotB and PotC involved in spermidine uptake activity. J Biol Chem 285:39061–39069

Igarashi K, Kashiwagi K (2010a) Characteristics of cellular polyamine transport in prokaryotes and eukaryotes. Plant Physiol Biochem 48:506–512

Igarashi K, Kashiwagi K (2010b) Modulation of cellular function by polyamines. Int J Biochem Cell Biol 42:39–51

Igarashi K, Kashiwagi K, Hamasaki H, Miura A, Kakegawa T, Hirose S, Matsuzaki S (1986) Formation of a compensatory polyamine by *Escherichia coli* polyamine-requiring mutants during growth in the absence of polyamines. J Bacteriol 166:128–134

Kashiwagi K, Hosokawa N, Furuchi T, Kobayashi H, Sasakawa C, Yoshikawa M, Igarashi K (1990) Isolation of polyamine transport-deficient mutants of *Escherichia coli* and cloning of the genes for polyamine transport proteins. J Biol Chem 265:20893–20897

Kashiwagi K, Suzuki T, Suzuki F, Furuchi T, Kobayashi H, Igarashi K (1991) Coexistence of the genes for putrescine transport protein and ornithine decarboxylase at 16 min on *Escherichia coli* chromosome. J Biol Chem 266:20922–20927

Kashiwagi K, Innami A, Zenda R, Tomitori H, Igarashi K (2002) The ATPase activity and the functional domain of PotA, a component of the sermidine-preferential uptake system in *Escherichia coli*. J Biol Chem 277:24212–24219

Kurihara S, Oda S, Tsuboi Y, Kim HG, Oshida M, Kumagai H, Suzuki H (2008) γ-Glutamylputrescine synthetase in the putrescine utilization pathway of *Escherichia coli* K-12. J Biol Chem 283:19981–19990

Kurihara S, Tsuboi Y, Oda S, Kim HG, Kumagai H, Suzuki H (2009) The putrescine Importer PuuP of *Escherichia coli* K-12. J Bacteriol 191:2776–2782

Lin HH, Hsu CC, Yang CD, Ju YW, Chen YP, Tseng CP (2011) Negative effect of glucose on *ompA* mRNA stability: a potential role of cyclic AMP in the repression of *hfq* in *Escherichia coli*. J Bacteriol 193:5833–5840

Marton LJ, Pegg AE (1995) Polyamines as targets for therapeutic intervention. Annu Rev Pharmacol Toxicol 35:55–91

Meng SY, Bennett GN (1992) Nucleotide sequence of the *Escherichia coli cad* operon: a system for neutralization of low extracellular pH. J Bacteriol 174:2659–2669

Neely MN, Olson ER (1996) Kinetics of expression of the *Escherichia coli cad* operon as a function of pH and lysine. J Bacteriol 178:5522–5528

Nemoto N, Kurihara S, Kitahara Y, Asada K, Kato K, Suzuki H (2012) Mechanism for regulation of the putrescine utilization pathway by the transcription factor PuuR in *Escherichia coli* K-12. J Bacteriol 194:3437–3447

Oliver DB, Beckwith J (1982) Regulation of a membrane component required for protein secretion in *Escherichia coli*. Cell 30:311–319

Pistocchi R, Kashiwagi K, Miyamoto S, Nukui E, Sadakata Y, Kobayashi H, Igarashi K (1993) Characteristics of the operon for a putrescine transport system that maps at 19 minutes on the *Escherichia coli* chromosome. J Biol Chem 268:146–152

Sambrook J, Russell DW (2001) Transformation of *E. coli* by electroporation. In: Sambrook J, Russell DW (eds) Molecular Cloning: A Laboratory Manual 3rd Ed. Cold Spring Harbor Laboratory, NY, pp 1.119–111.122

Shimada T, Fujita N, Yamamoto K, Ishihama A (2011) Novel roles of cAMP receptor protein (CRP) in regulation of transport and metabolism of carbon sources. PLoS One 6:e20081

Soksawatmaekhin W, Kuraishi A, Sakata K, Kashiwagi K, Igarashi K (2004) Excretion and uptake of cadaverine by CadB and its physiological functions in *Escherichia coli*. Mol Microbiol 51:1401–1412

Takayama M, Ohyama T, Igarashi K, Kobayashi H (1994) *Escherichia coli cad* operon functions as a supplier of carbon dioxide. Mol Microbiol 11:913–918

Terui Y, Higashi K, Taniguchi S, Shigemasa A, Nishimura K, Yamamoto K, Kashiwagi K, Ishihama A, Igarashi K (2007) Enhancement of the synthesis of RpoN, Cra, and H-NS by polyamines at the level of translation in *Escherichia coli* cultured with glucose and glutamate. J Bacteriol 189:2359–2368

Tomitori H, Kashiwagi K, Igarashi K (2012) Structure and function of polyamine-amino acid antiporters CadB and PotE in *Escherichia coli*. Amino Acids 42:733–740

Watson N, Dunyak DS, Rosey EL, Slonczewski JL, Olson ER (1992) Identification of elements involved in transcriptional regulation of the *Escherichia coli cad* operon by external pH. J Bacteriol 174:530–540

Yoshida M, Kashiwagi K, Kawai G, Ishihama A, Igarashi K (2001) Polyamine enhancement of the synthesis of adenylate cyclase at the translational level and the consequential stimulation of the synthesis of the RNA polymerase σ^{28} subunit. J Biol Chem 276:16289–16295